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14. ABSTRACT

The DNA-dependent protein kinase (DNA-PK) plays a very important role in the repair of DNA double-strand breaks generated by ionizing radiation (IR). The activation of DNA-PK in response to IR involves multiple autophosphorylations at S/TQ residues of the catalytic subunit, DNA-Pkcs. We find that the activation of DNA-PKcs is attenuated during the S/G2 phases of the cell cycle, phases during which Brca1 is expressed. We found that DNA-Pkcs interacts with Brca1 and have mapped the interaction domain of Brca1. In order to investigate if the interaction of Brca1 with DNA-PKcs might attenuate the activation of the latter, we examined DNA-PKcs autophosphorylation in Brca1-deficient HCC1937 cells ectopically expressing Brca1. We, however, failed to observe any significant differences in DNA-Pkcs autophosphorylation in the presence or absence of Brca1 in these cells. The lack of differences could be due to the low levels of Brca1 expression in these cells. Overexpression of the DNA-Pkcs-interaction domain of Brca1 might help to circumvent this problem.

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INTRODUCTION

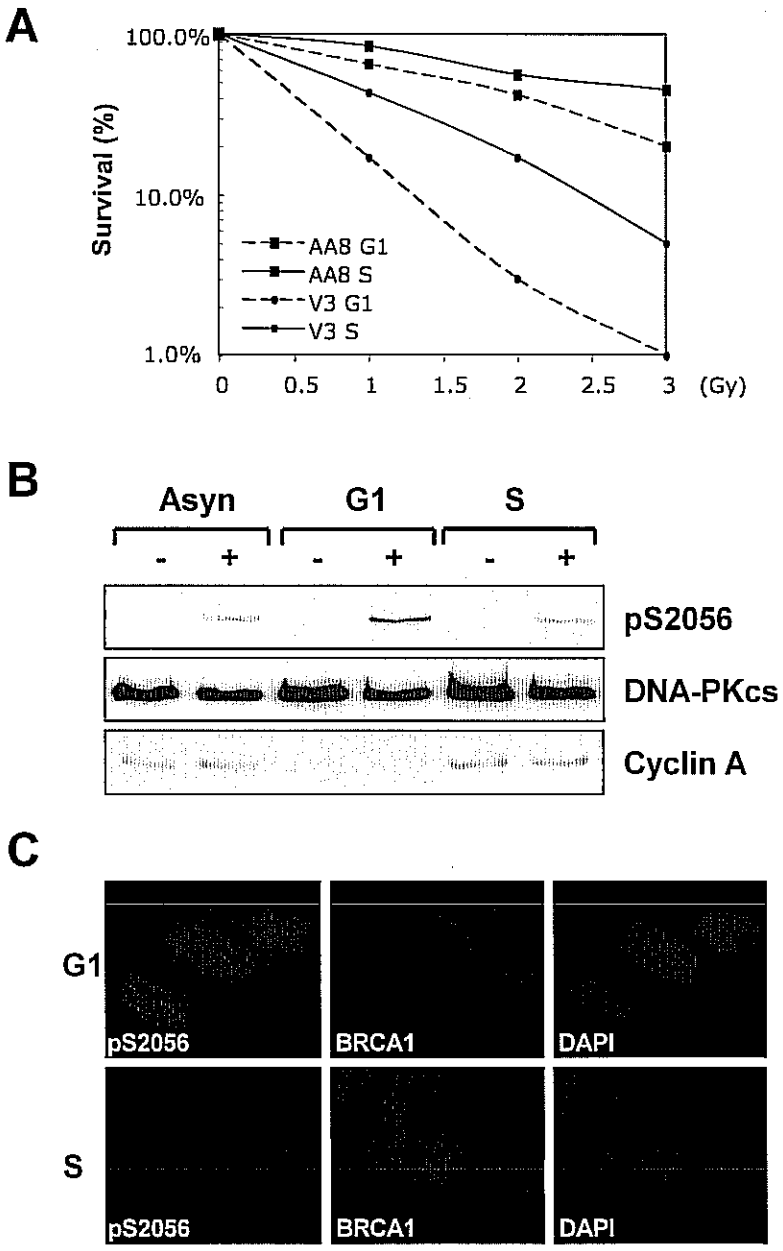
The DNA-dependent protein kinase (DNA-PK), consisting of a catalytic subunit DNA-PKcs and a DNA binding component Ku, plays a critical role in DNA double-strand break repair and in V(D)J recombination. DNA-PK also plays a very important role in triggering apoptosis in response to severe DNA damage or critically shortened telomeres. Components of the DNA-PK complex are also present at the mammalian telomere where they function in capping chromosome ends. In addition, DNA-PK appears to be involved in mounting an innate immune response to bacterial DNA and to viral infection. For a detailed review see (1).

The most important role of DNA-PK is in the repair of DNA double-strand breaks (DSBs) by the non-homologous end rejoining (NHEJ) pathway. Of the various types of DNA damage that arise within the cell, DNA double-strand breaks (DSBs) are particularly dangerous as they can lead to cell death or cancer if improperly repaired. Recently, our group, among others, discovered that that the activation of DNA-PKcs in response to DNA damage involves its autophosphorylation at several S/TQ residues (1). Phospho-specific antibodies recognizing these autophosphorylation sites can thus be used to accurately monitor the activation of DNA-PK within the cell as well as its localization. Using phospho-specific DNA-PKcs antibodies we found that the activation of DNA-PKcs in response to ionizing radiation (IR) is suppressed in the S/G2 phase of the cell division cycle. We also found that DNA-PKcs interacted with the tumor suppressor Brca1 whose expression is high during the S/G2 phases (2). We are interested in further dissecting this interaction and determining if Brca1 might modulate the activaton of DNA-PK in response to IR.

BODY

1) The activation of DNA-PK and the repair of DNA breaks by the NHEJ pathway is regulated in a cell cycle-dependent manner. It has been proposed that NHEJ and HR may be differentially regulated throughout the cell cycle with NHEJ playing a major role in G1/early S phases and HR playing a major role in late S/G2 phases when a sister chromatid is available (3). Indeed, DNA-PKcs-deficient V3 cells are less sensitive to IR in the S phase of the cell cycle as compared to G1 indicating that HR may partly compensate for NHEJ-deficiency in S phase cells (Fig. 1a). In order to directly monitor the phosphorylation of DNA-PK in different phases of the cell cycle, normal human skin fibroblasts (HSF) synchronized in G1 or S phases were irradiated and DNA-PKcs phosphorylation was analyzed. As shown in Fig. 1b, the protein level of DNA-PKcs remains constant throughout the cell cycle, whereas IR-induced S2056 autophosphorylation decreases from G1 phase to S phase. The decrease of IR-induced DNA-PKcs phosphorylation in S phase was also evident by fluorescent immunostaining with anti-S2056 antibody (Fig. 1c) and with anti-T2609 antibody (Fig. 1d) suggesting an overall reduction of IR-induced DNA-PKcs phosphorylation in the S phase of the cell cycle. Please note that the G1 cells stain poorly for Brca1 while the S phase cells stain strongly for Brca1 thereby confirming their cell cycle distribution (4). These results indicate that phosphorylation of DNA-PK in response to IR is regulated in a cell cycle-dependent manner. In HSF cells synchronized in G1 phase, greater than 90% of the HSF cells were positive for S2056 foci upon IR. In contrast, less than 15% of the HSF cells synchronized in S phase were positive for S2056 foci (Fig. 1e).

Figure 1


A
100.0%
10.0%
1.0%
Survival (%)
AA8 G1
AA8 S
V3 G1
V3 S
0
0.5
1
1.5
2
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(Gy)
B
Asyn
G1
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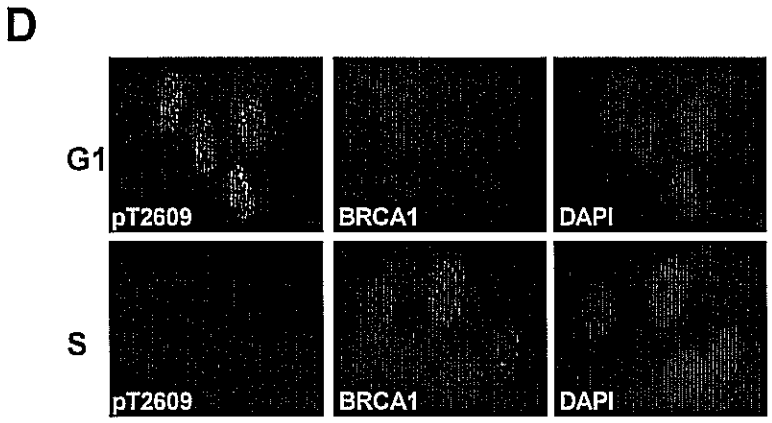


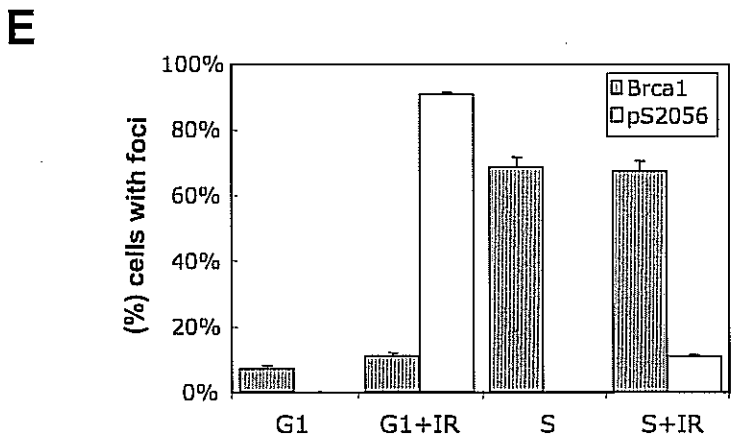


Fig. 1. Decrease of IR-induced S2056 and T2609 phosphorylation in the S phase. (a) V3 and the parental AA8 cells, synchronized in G1phase or in S phase, were irradiated at the indicated doses, and were plated out for analyzing their colony-forming abilities. **(b)** Asynchronized and synchronized HSFs were irradiated (10 Gy, 30 min recovery). Nuclear extracts were western blotted with anti-pS2056 antibody, anti-DNA-PKcs antibody, or anti-Cyclin A antibody. **(c, d)** HSFs synchronized in G1 or S phases were irradiated (10 Gy, 30 min recovery) and were co-immunostained with anti-pS2056/anti-Brca1 antibodies **(c)**, or anti-pT2609/anti-Brca1 antibodies **(d)**. **(e)** HSFs were scored for positive staining with anti-pS2056 or anti-Brca1 antibodies. More than 200 nuclei were counted in each experiment and the result is the average of two independent experiments.

2. DNA-PKcs constitutively interacts with Brca1. As DNA-PKcs activation is attenuated in the S/G2 phase of the cell cycle (see above), and because Brca1 is expressed in the S/G2 phases, we wanted to see if any regulatory interaction might exist between these two proteins. Towards this end, nuclear extracts were prepared from mock-irradiated and irradiated (10 Gy, 30 min) HeLa cells and the extracts were immunoprecipitated (ip) with anti-Brca1 or anti-DNA-PKcs antibodies and the immunoprecipitates were analyzed by Western blotting. Interestingly, we found that DNA-PKcs co-immunoprecipitated in the Brca1 ip (Fig. 2a). Conversely, we also found that Brca1 co-immunoprecipitated in a DNA-PKcs ip (Fig. 2b). The interaction between DNA-PKcs and Brca1 appeared to be constitutive and did not change upon irradiation of the cells.

Figure 2

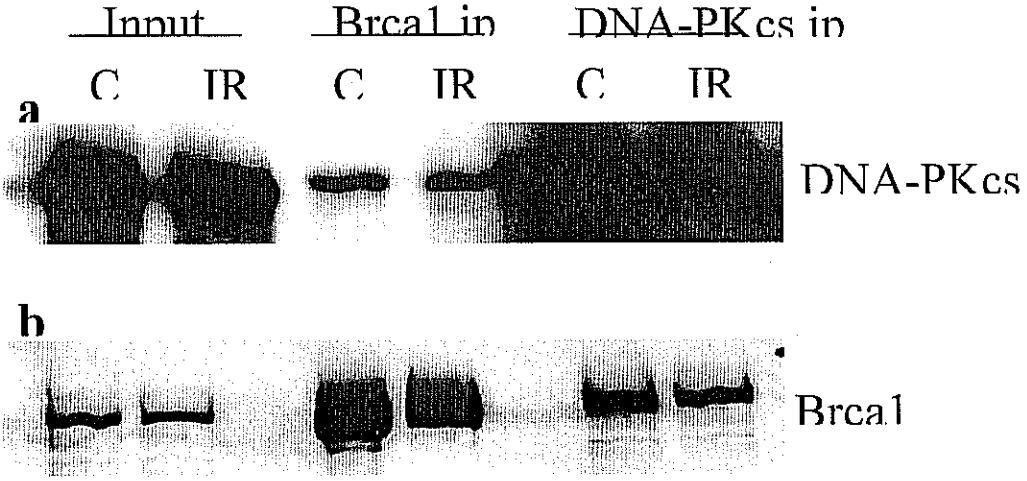
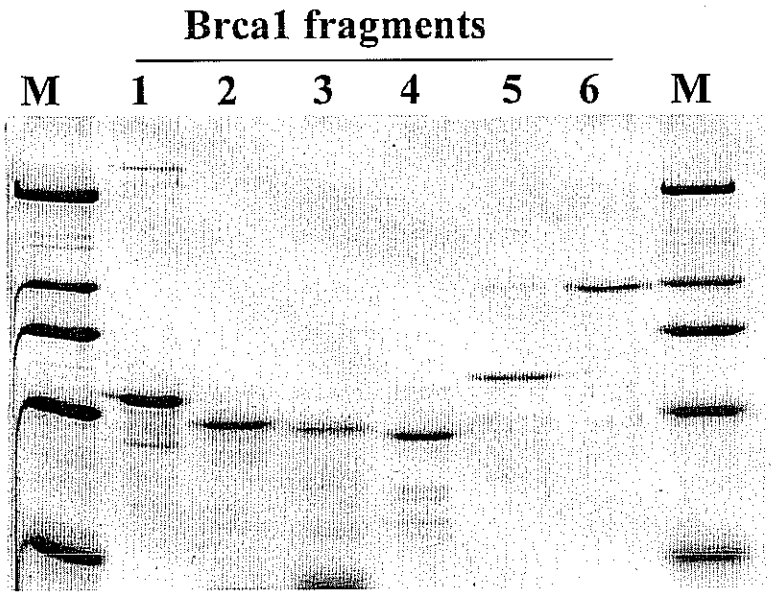


Fig. 2. DNA-PKcs co-immunoprecipitates with Brca1. Mock-irradiated (C) or irradiated (IR) HeLa nuclear extracts were immunoprecipitated with anti-Brca1 antibody or anti-DNA-PKcs antibody as indicated and the immunoprecipitates were analyzed by Western blotting with anti-DNA-PKcs antibody (a) or anti-Brca1 antibody (b).

3. Mapping of the DNA-PKcs-interaction domain of Brca1. In order to dissect the interaction of Brca1 with DNA-PKcs, we wanted to first determine the DNA-PKcs-interaction region of Brca1. We expressed overlapping fragments of Brca1 (5) as GST-fusion proteins in E.coli. (Fig. 3a). These fragments were used in GST pull down assays of purified DNA-PK complexes in the presence or absence of DNA. We found that Brca1 fragment 2 (aa 260-553) and fragment 3 (aa 502-802) interacted with DNA-PKcs while the remaining fragments did not (Fig. 3b). The pull down of DNA-PK by these two fragments was confirmed by Western blotting with anti-Ku70/80 antibodies. The addition of sheared salmon sperm DNA had no effect on the interaction.

Figure 3

a



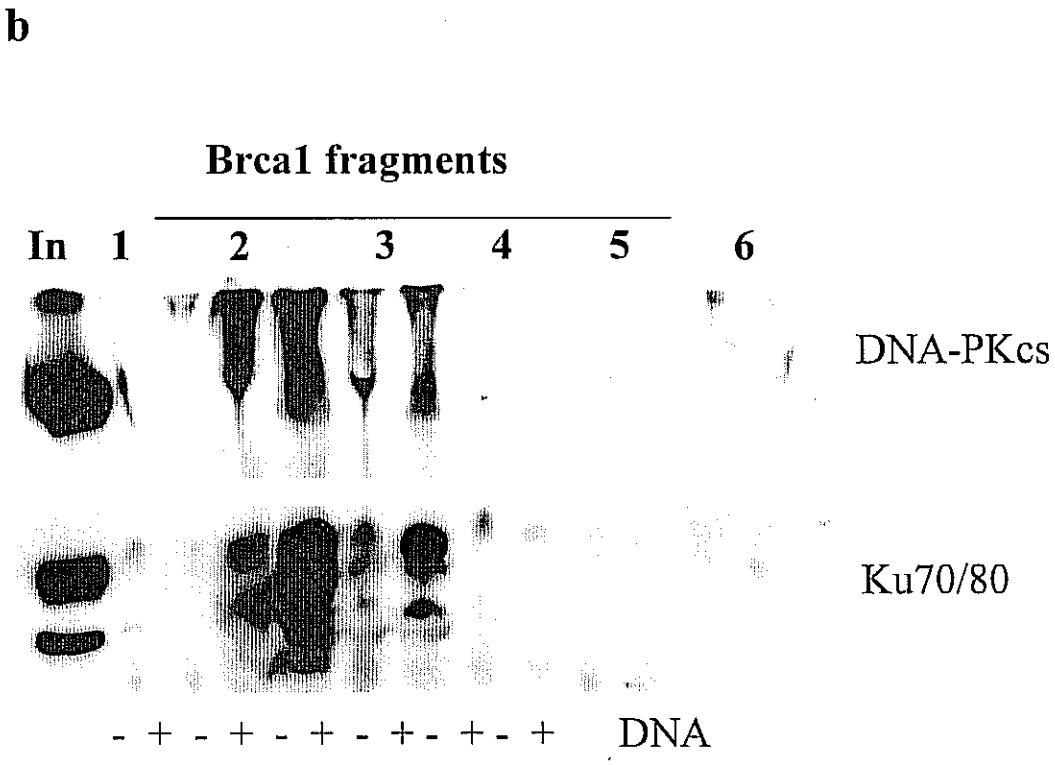


Fig. 3 (a) Expression of Brca1 fragments 1-6 (4) as GST-fusion proteins. **(b)** GST pulldown of DNA-PK components (DNA-PKcs and Ku70/80) by Brca1 fragments 2 and 3.

4. Effect of ectopic Brca1 expression on DNA-PKcs autophosphorylation. We have carried out preliminary experiments to investigate if the interaction of Brca1 with DNA-PKcs might modulate the activation of DNA-PKcs upon DNA damage. Brca1-deficient HCC1937 cells and HCC1937 cells ectopically expressing Brca1 (6) were irradiated with X-rays (10 Gy, 30 min) and the activation of DNA-PK examined by immunofluorescence staining with phospho-specific DNA-PKcs antibodies. We found that DNA-PKcs was autophosphorylated (at S2056 and T2609) and capable of forming radiation induced foci even in cells expressing Brca1 (Fig. 4). Therefore, it appears that DNA-PKcs activation may not be significantly suppressed by Brca1 alone. Alternately, it is also possible that the levels of ectopic Brca1 might not be sufficiently high to regulate DNA-PKcs which is

highly abundant in human cells. We are currently in the process of generating cells overexpressing fragments 2 and 3 of Brca1 to see if these DNA-PKcs-interacting regions might have a modulatory effect on DNA-PK activation.

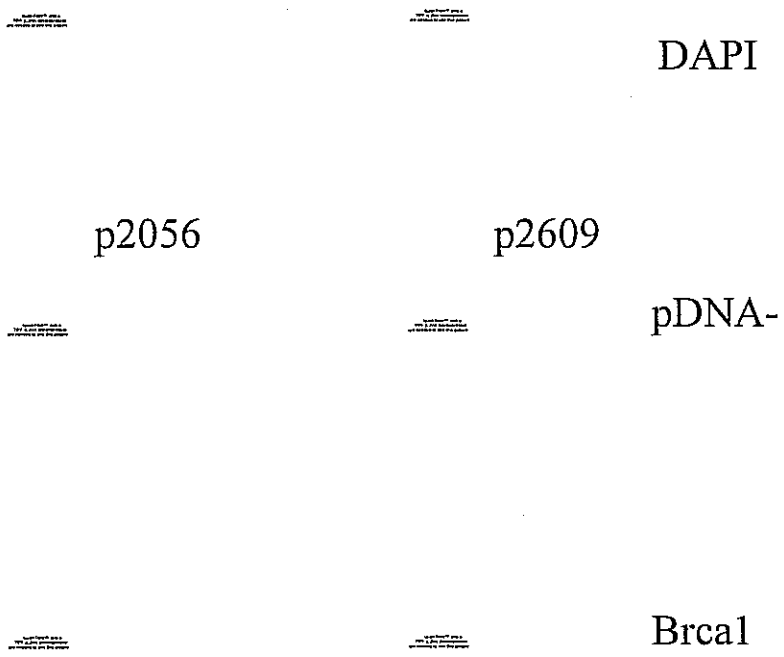


Fig. 4. DNA-PKcs autophosphorylation (at S2056 and T2609) and focus formation in reponse to IR in HCC1937 cells ectopically expressing Brca1.

5. Effect of DNA-PKcs on the phosphorylation and activation of Brca1. We are also interested in examining if the interaction of DNA-PKcs with Brca1 might serve to

modulate IR-induced Brca1 phosphorylation and functions. We propose to first examine if Brca1 phosphorylation in response to IR is affected in cells deficient in DNA-PKcs. We have raised and purified phospho-specific antibodies against the phosphorylation sites on Brca1 that are modified in response to IR (serines 1387, 1423, 1524 (7)) (Fig. 5a). These antibodies can be used to visualize the phosphorylation of Brca1 in response to IR (Fig. 5b). We propose to use these antibodies to analyze if DNA-PKcs might modulate the modification of Brca1upon IR.

Figure 5

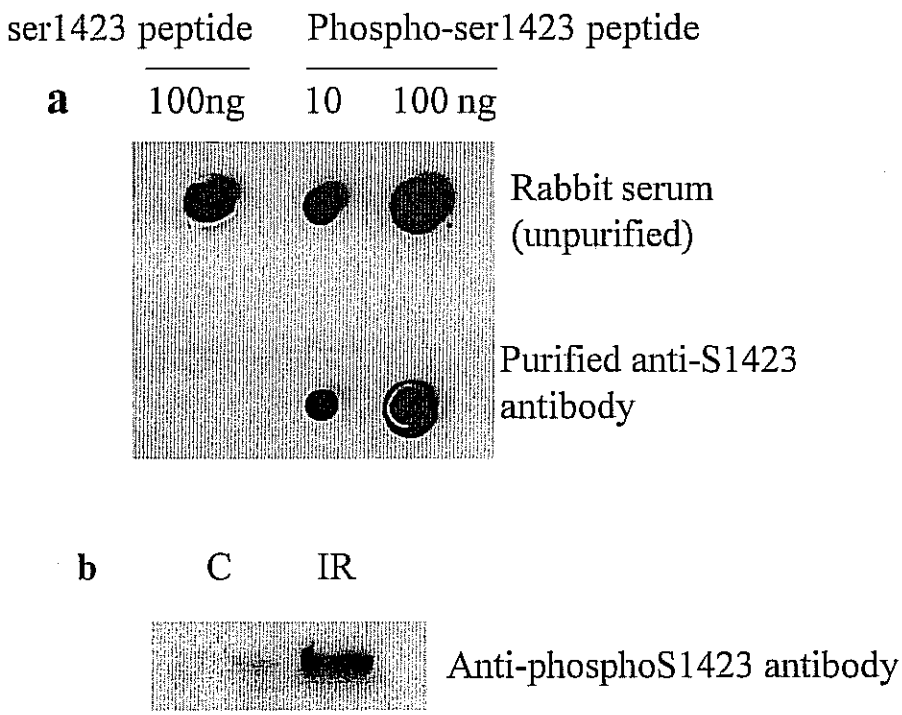


Fig. 5. a. Rabbit polyclonal antibodies were raised against peptides with phospho-S1423 site of Brca1, purified over a phospho-peptide column, and tested by dot blotting against unphosphorylated and phosphorylated peptides. **B.** The pS1423 antibody was used to visualize the phosphorylation of Brca1 upon irradiation of HeLa cells by Western blotting.

KEY RESEARCH ACCOMPLISHMENTS

1. The activation of DNA-PK is attenuated in the S/G2 phases of the cell cycle.

2. DNA-PK interacts with Brca1 and the interaction is not affected upon ionizing radiation.
3. The DNA-PK-interaction region of Brca1 lies between aa 260-553.
4. The phosphorylation and DNA damage-site localization of DNA-PKcs in cells ectopically expressing Brca1 was investigated. Experiments are underway to investigate the effect of overexpressing Brca1 fragments on DNA-PK activation.
5. Phospho-specific Brca1 antibodies were raised and will be used to investigate the influence of DNA-PKcs on Brca1 phosphorylation and functions upon irradiation.

REPORTABLE OUTCOMES

Published Manuscripts

1] DNA-PK phosphorylates histone H2AX during apoptotic DNA fragmentation in mammalian cells.
B. Mukherjee, C. Kessinger, J. Kobayashi, B.P. Chen, D.J. Chen, A. Chatterje, and S. Burma
DNA Repair 5:575-590 (2006)

2] Cell cycle dependence of DNA-PK phosphorylation in response to DNA double-strand breaks. B. Chen, D.W. Chan, J. Kobayashi, S. Burma, A. Asaithamby, K. Morotomi-Yano, E. Botvinick, J. Qin, and D.J. Chen
Journal of Biological Chemistry 280:14709-14715 (2005)

3] Gene expression profiles of normal human fibroblasts after ionizing radiation: a comparative study with low and high doses. L.-H. Ding, M. Shingyoji, F. Chen, J.-J. Hwang, S. Burma, J.-F. Cheng, and D. J. Chen
Radiation Research 164:17-26 (2005)

Meeting Abstracts;

1] Phosphorylation of DNA-PK and DNA-double strand break repair.
B. Chen, J. Kobayashi, S. Burma, D. Chan, A. Asaithamby, J. Qin, and D.J. Chen
In abstract of the AACR Special Conference on Radiation Biology and Cancer (2004)

2] Differential requirement of DNA-PKcs in S phase for double-strand break repair.
B. Chen, J. Kobayashi, S. Burma, D. Chan, A. Asaithamby, J. Qin, and D.J. Chen
In abstract of the Radiation Research Society Annual Meeting (2004)

3] Autophosphorylation of the DNA-dependent protein kinase is required for rejoining of DNA double-strand breaks.
B. Chen, J. Kobayashi, S. Burma, D. Chan, K. Yano, J. Qin, and D.J. Chen
In abstract of the International Workshop on Ataxia-Telangiectasia (2003)

CONCLUSIONS

We were interested in investigating whether the interaction of DNA-PKcs and Brca1, as observed by us, had any functional consequences. We were especially interested in determining if Brca1 might be responsible for the attenuated DNA-PKcs activation in

S/G2 phases that we observed. We found that the ectopic expression of Brca1did not significantly affect DNA-PK activation and localization to damage sites. However, this could also be because the levels of ectopic Brca1 in the cell lines used might not have been sufficiently high. We are currently trying to overexpress the DNA-PKcs-interaction domain of Brca1 to see if DNA-PK activation is affected. We have also raised antibodies recognizing Brca1 phosphorylated at specific sites and will use these to investigate if the interaction of DNA-PKcs and Brca1 might modulate Brca1 phosphorylation upon DNA damage induction.

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7. Gatei, M., Zhou, B. B., Hobson, K., Scott, S., Young, D., and Khanna, K. K. Ataxia telangiectasia mutated (ATM) kinase and ATM and Rad3 related kinase mediate phosphorylation of Brca1 at distinct and overlapping sites. In vivo assessment using phospho-specific antibodies. J Biol Chem, *276:* 17276-17280, 2001.